




MLKL Aggravates Ox-LDL-Induced Cell Pyroptosis via Activation of NLRP3 Inflammasome in Human Umbilical Vein Endothelial Cells

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Abstract— Atherosclerosis is a progressive chronic inflammation in the arterial walls. It is believed that the deposition of low-density lipoprotein (LDL) and its damage to endothelial cells play a vital role in atherosclerosis. Oxidized LDL (Ox-LDL) was confirmed to induce endothelial cell pyroptosis which plays an important role in intima inflammation and the development of atherosclerosis, but the underlying molecular mechanism needs to be explored. Here, we showed that ox-LDL upregulated the expression of mixed lineage kinase domain-like (MLKL) protein at both the mRNA and protein levels in endothelial cells, associated with the augment of pro-caspase-1 cleavage, interleukin-1 β (IL-1 β) maturation, pro-IL-1 β production, and lactate dehydrogenase (LDH) release. Overexpression of MLKL substantially aggravated ox-LDL-induced increasing levels of caspase-1, IL-1 β , pro-IL-1 β , and LDH. MLKL-induced caspase-1 activation and IL-1 β maturation were abolished by NLR family, pyrin domain-containing 3 (NLRP3) specific inhibitor MCC950, or extracellular high potassium concentration. Our findings indicated that MLKL is essential for regulation of ox-LDL-induced pyroptosis and inflammation through the activation of NLRP3 inflammasome, and suggested that MLKL could act as potential therapeutic targets to ameliorate atherosclerosis-related diseases.

KEY WORDS: Ox-LDL; MLKL; pyroptosis; NLRP3; caspase-1; IL-1 β .

INTRODUCTION

Coronary atherosclerosis is the basis of most coronary artery disease (CAD) and leads to the development of

many other cardiovascular diseases [1]. The pathogenesis of atherosclerosis is complex and still not fully understood. It is now well accepted that atherosclerosis is a chronic inflammatory disease within the vascular walls. It always

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begins with the damage to the endothelial. After endothelial injury, low-density lipoprotein (LDL) deposits in vascular intima and is modified to oxidized LDL [2, 3]. Ox-LDL can activate endothelial cells, leading to increased expression of adhesion molecules and release of inflammatory cytokines [1, 4].

Pyroptosis is a novel form of programmed necrosis, which is a unique form and mechanism of programmed cell death compared with apoptosis and autophagic cell death [5]. Mechanically, pyroptosis is mainly driven by caspase-1-mediated signaling pathway. In this canonical pyroptosis signaling pathway, pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) are detected by a variety of inflammasomes, such as NLR family and pyrin domain-containing 3 (NLRP3) inflammasome, which can be activated by a range of stimuli. The NLRP3 inflammasome is currently the best characterized inflammasome and consists of NLRP3, apoptosis-associated speck-like protein containing a CARD (ASC), and caspase-1. Upon NLRP3 activation, inactive procaspase-1 cleaves into p20 and p10 subunits that form the active caspase-1, which then leads to maturation and secretion of interleukin-1 β (IL-1 β) and interleukin-18 (IL-18) [6]. In addition to processing pro-inflammatory cytokines, active caspase-1 further leads to plasma membrane rupture, cytoplasmic swelling, DNA fragmentation, and the release of pro-inflammatory cellular contents [7, 8].

Recent studies have shown that pyroptosis is associated with a variety of cardiovascular diseases [9]. Endothelial cell pyroptosis may lead to a series of downstream events of atherosclerosis. In endothelial cells, the caspase-1-mediated inflammasome pathway senses lipid accumulation and inflammatory mediators, and triggers pyroptosis [10–12]. Caspase-1 activation promotes endothelial activation. The expression levels of endothelial cell adhesion molecules were increased, and thus trigger mononuclear cell adhesion [13]. Endothelial cell pyroptosis may lead to reduced endothelial cell quantity and integrity, increasing endothelium permeability, and thus promoting smooth muscle cell migration and deposition [14]. Most importantly, pyroptosis of endothelial cells leads to the release of pro-inflammatory cytokines, such as IL-1 β and IL-18, leading to vascular inflammation. As we all know, atherosclerosis is a lipid-driven inflammatory disease of the arterial intima in which the balance of pro-inflammatory and inflammation-resolving mechanisms dictates the final clinical

outcome [15]. The role of inflammation caused by pyroptosis in atherosclerosis cannot be ignored.

Mixed lineage kinase domain-like (MLKL) protein is known as the executor of necroptosis [16]. Necroptosis is defined by activation of the receptor-interacting protein kinase (RIPK)1 and RIPK3 to form an oligomeric necrosome, which leads to the phosphorylation and activation of the effector pseudokinase MLKL [16, 17]. Once activated, MLKL multimerizes and translocates to the cell membrane, where it triggers ion release *via* pore formation, leading to cell swelling and rupture [18, 19]. MLKL has been found to be associated with pathological conditions like cancer, neurodegenerative diseases, and inflammatory diseases [20, 21]. Our previous findings showed that MLKL was significantly upregulated by ox-LDL in endothelial cells (ECs), smooth muscle cells (SMCs), and human myeloid leukemia mononuclear (THP-1) cells, and participated in the progress of atherosclerosis through aggravating inflammatory responses and inhibiting autophagy flux [22].

In this study, the role of MLKL in ox-LDL-triggered NLRP3 inflammasome activation in endothelial cells was studied and the possible mechanisms were also investigated.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Gibco (Grand Island Biological Company, New York, NY, USA). Antibodies against rabbit MLKL (A5579), NLRP3 (A5652), and IL-1 β (A1112) were obtained from Abclonal (Woburn, MA, USA). Antibodies against mouse caspase-1 (sc-398715) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). MCC950 (CP-456773) was purchased from Selleck Chemicals (Shanghai, China).

Ox-LDL Preparation

The ox-LDL was derived from human low-density lipoprotein (LDL). At 37 °C, 2 mM CuSO₄ was added overnight to oxidize (ox) the LDL, and oxidation was stopped with an excess of sodium EDTA. Agarose gel electrophoresis was used to analyze the migration of LDL. The colorimetric determination of thiobarbituric acid

reactive species used malondialdehyde (MDA) as a standard, at 12.0 nM MDA per mg protein for the ox-LDL.

Cell Culture

Human umbilical vein endothelial cells (ATCC CRL-1730) were obtained from American Type Culture Collection. ECs were cultured in DMEM with 10% fetal bovine serum. Cells were incubated at 37 °C in an atmosphere of 5% CO₂. Cells were seeded in 6- or 12-well plates or 60-mm dishes and grown to 70–80% confluence before use.

QRT-PCR

Total RNA from cultured cells and tissues was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed. Real-time PCR was performed on the lightCycler 480 II (Roche, Pleasanton, CA, USA) with SYBR Green Dye detection (TaKaRa Bio, Mountain View, CA, USA). All samples were assayed in triplicate. The data were analyzed using the $\Delta\Delta C_t$ method, with GAPDH as a reference in the mRNA analysis. The primer sense and antisense sequences were as follows: MLKL: forward 5'-AGGAGGCTAATGGGGAGATAGA-3', reverse 5'-TGGCTTGCTGTTAGAAACCTG-3'; GAPDH: forward 5'-GCACCGTCAAGGCTGAGAAC-3', reverse 5'-TGGTGAAGACGCCAGTGGA-3'.

Immunoblot Analyses

The total protein was measured by the bicinchoninic acid protein assay kit (P0010-1; Beyotime, China). The cleavage products of each sample were separated by 12.5% SDS-PAGE. The western blots were incubated for 8 h with 1:1000-diluted primary antibodies, and then incubated 2 h at room temperature with horseradish-peroxidase-conjugated secondary antibodies. Visualization of proteins used chemiluminescence (ECL Plus Western Blot Detection System; Amersham Biosciences, Foster City, CA, USA).

Lactate Dehydrogenase Release

Cell culture supernatants were collected. LDH levels in the supernatants were determined using an LDH cytotoxicity detection kit (Dojindo Laboratories, Japan) according to the manufacturer's protocol. The absorbance was read at 490 nm with a microplate reader.

Recombinant Plasmid Construction

A plasmid containing the full-length MLKL cDNA was purchased from OriGene (Rockville, MD, USA). MLKL cDNA was amplified by PCR and subcloned into the pcDNA3.1(+) vector. The correct sequence of the MLKL cDNA in the recombinant plasmid was verified by sequencing and named pcDNA-MLKL. The plasmid was used to transfect ECs using Lipofectamine 3000 transfection reagent.

siRNA Assay

Negative control siRNA (si-control) and siRNA-MLKL (si-MLKL) were purchased from Ribo Targets (Guangzhou, China). ECs and SMCs were transfected with 50 nM siRNA. Control samples for all experimental procedures were processed with a non-targeting control mimic sequence of equal concentration. Western blotting was performed to observe the efficiency of siRNA protein knockdown.

Statistical Analysis

Data were analyzed using the SPSS version 13.0 (SPSS, Chicago, IL, USA) software. Data were presented as the mean \pm SD or median (interquartile range) unless otherwise indicated. The results were analyzed by one-way analysis of variance or unpaired Student's *t* tests when continuous variables were normally distributed. A two-tailed *P* value < 0.05 was considered statistically significant.

RESULTS

Ox-LDL Upregulated MLKL Expression and Induced Pyroptosis in ECs

Our previous study found that MLKL was significantly increased by ox-LDL in THP-1 macrophages stimulated with ox-LDL by microarray analysis [22]. In order to verify the effects of ox-LDL on MLKL in ECs, we carried out qRT-PCR and western blot to determine the expression of MLKL in ECs treated with different concentrations of ox-LDL for 24 h. The expression of total MLKL and phosphorylated MLKL was significantly increased at both mRNA and protein levels (Fig. 1a and b).

Recent studies have shown that pyroptosis is involved in inflammatory reactions. It is noteworthy that pyroptosis could exacerbate the progression of atherosclerosis. To

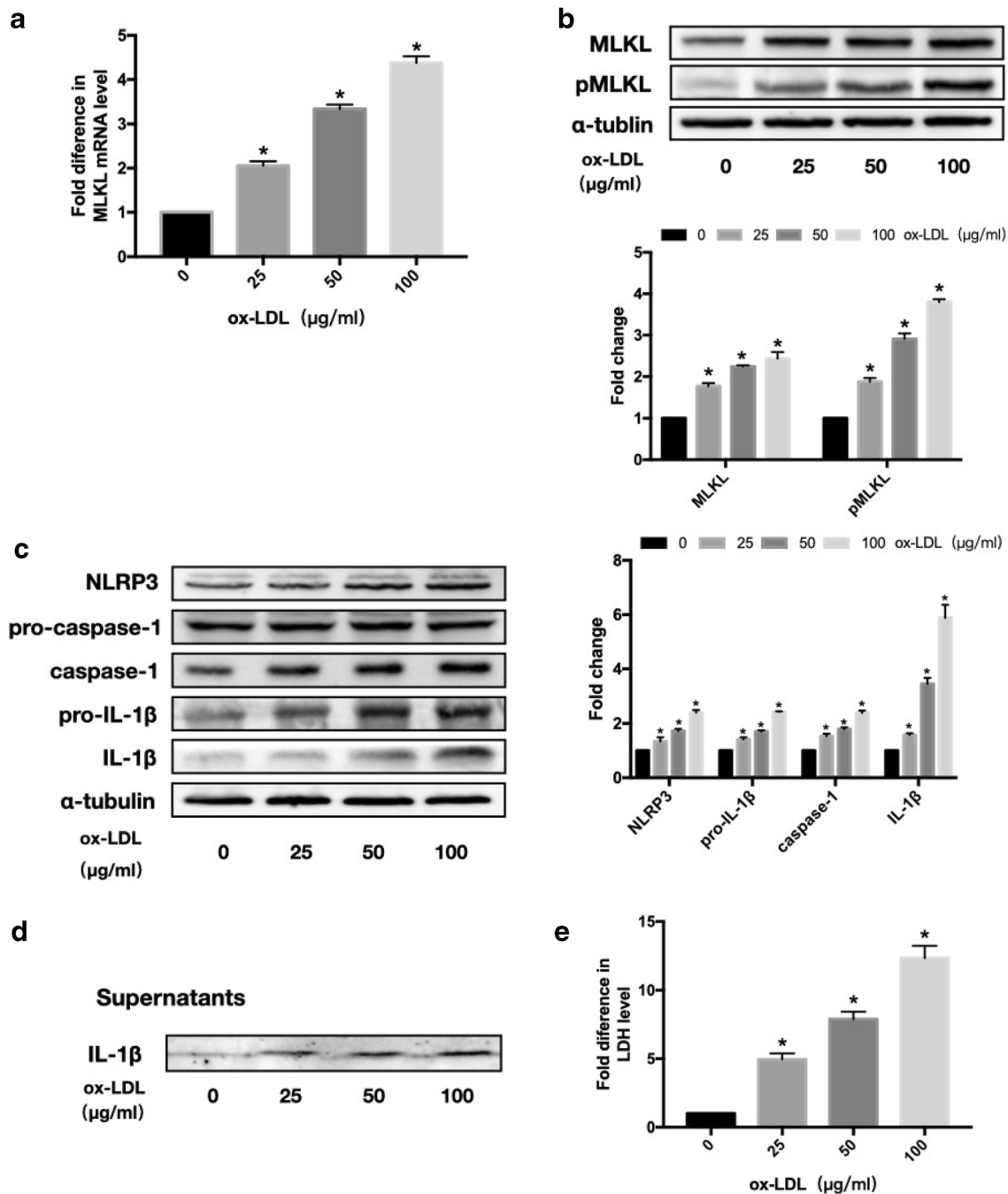


Fig. 1. Ox-LDL upregulated MLKL expression and induced pyroptosis in ECs. ECs were treated with ox-LDL (0, 25, 50, or 100 mg/l) for 24 h. **a** The expression of MLKL was determined by qRT-PCR. **b** Total MLKL and phosphorylated MLKL protein levels were measured by western blotting. **c** The expression of NLRP3, caspase-1, and IL-1β were measured by western blotting. **d** Proteins released to culture media were enriched by ultrafiltration centrifugation. The levels of matured IL-1β in the supernatants of the cell culture media were analyzed by western blotting. **e** The levels of LDH release in the supernatants of the cell culture media were analyzed. **P* < 0.05. Data are represented as mean ± SD values from 3 independent experiments with each experiment done in triplicate.

observe the effect of ox-LDL on pyroptosis in ECs, cells were treated with ox-LDL in different concentrations for 24 h. The protein levels of pro-caspase-1, caspase-1, pro-IL-1 β , and IL-1 β were determined, as well as the levels of secreted IL-1 β in culture media. And LDH release assay was carried out to measure the release of cell content caused by pyroptosis. The results showed, as in Fig. 1, that the expressions of pro-caspase-1 is stable, but ox-LDL could induce NLRP3 upregulation, caspase-1 activation, pro-IL-1 β production, IL-1 β maturation and release (Fig. 1c and d), and the release of LDH (Fig. 1e). These data suggest that ox-LDL induced cell pyroptosis in ECs.

MLKL Triggered Ox-LDL-Induced Pyroptosis in ECs

Previous studies have shown that MLKL plays a crucial role in many kinds of inflammatory diseases [23–26]. We investigated whether MLKL is involved in ox-LDL-mediated pyroptosis. Firstly we treated ECs with pcDNA-MLKL to upregulate the expression of MLKL, and stimulated cells with 50 mg/ml ox-LDL for 24 h. Western blot analysis showed that MLKL upregulation aggravates ox-LDL-induced caspase-1 cleavage (Fig. 2a). The release of LDH was also increased after MLKL upregulation (Fig. 2c). Next, knockdown of MLKL reversed the increased levels of caspase-1, pro-IL-1 β , and IL-1 β caused by ox-LDL (Fig. 2b). The release of LDH caused by ox-LDL was also blocked by MLKL knockdown (Fig. 2d). Collectively, these data indicated that MLKL is involved in ox-LDL-induced pyroptosis.

MLKL Triggered Ox-LDL-Induced Pyroptosis via NLRP3 Inflammasome Activation

The canonical pyroptosis signaling pathway requires recognition to cell damage signals and assembly of inflammasomes. There are different kinds of inflammasomes that would lead to cell pyroptosis. Our experiment results showed that ox-LDL induced NLRP3 expression, and MLKL upregulation further increased NLRP3 protein level (Fig. 3a). And ox-LDL-induced NLRP3 upregulation was significantly suppressed by MLKL knockdown (Fig. 3b). To confirm whether MLKL triggered ox-LDL-induced pyroptosis via NLRP3 inflammasome activation, we used a specific NLRP3 inhibitor MCC950. Western blot analysis showed that after inhibiting activation of NLRP3 protein, the maturation of caspase-1 and IL-1 β induced by ox-LDL were abolished, as well as MLKL triggered ox-LDL-induced maturation of caspase-1 and IL-1 β (Fig. 3c), which means MLKL triggered ox-LDL-induced pyroptosis via NLRP3 inflammasome.

NLRP3 could be activated by lysosomal enzyme cathepsin B, potassium efflux, reactive oxygen species (ROS), and other cell damage signals. Among them, potassium efflux is a common step that is necessary to stimulate the activation of NLRP3 inflammatory. As we have mentioned, phosphorylated MLKL translocated to the plasma membrane where it forms cation channels that are permeable potassium. To explore whether MLKL triggers ox-LDL-induced NLRP3 inflammasome assembly through potassium efflux, exogenous potassium chloride was added into cell culture media to inhibit potassium efflux. The cleavage of caspase-1 and IL-1 β was determined and the results showed that high level of extracellular potassium blocks the activation of NLRP3 inflammasome triggered by MLKL (Fig. 3d). Collectively, our findings showed that MLKL processed intracellular potassium efflux, and further triggered ox-LDL-induced pyroptosis via NLRP3 inflammasome activation.

DISCUSSION

Modern hypotheses introduce atherosclerosis as an inflammatory/lipid-based disease and NLRP3 inflammasome has been considered a link between lipid metabolism and inflammation because crystalline cholesterol and ox-LDL (two abundant components in atherosclerotic plaques) activate NLRP3 inflammasome [27]. Our findings showed that MLKL, an executor of necrosis, triggered ox-LDL-induced endothelial cell pyroptosis via NLRP3 inflammasome activation.

Cell death can be observed in atherosclerosis and plays an important role in the occurrence and development of atherosclerosis lesions [28]. Pyroptosis is involved in the formation and development of atherosclerosis by promoting the release of inflammatory cytokines, which is closely related to the stability of plaques [29]. Ox-LDL can induce macrophage pyroptosis, which plays an important role in the formation of atherosclerosis and the stability of plaques [30]. It has been reported that ox-LDL and cholesterol crystals in the necrotic areas of plaque increase the levels of NLRP3, pro-IL-1 β , and caspase-1, inducing cell pyroptosis [27, 31–33]. This phenomenon leads to the release of IL-1 β , which also aggravate the inflammatory response and play the role of atherosclerosis. Our finding shows that ox-LDL upregulates the pro-IL-1 β levels and activates NLRP3 inflammasome which enables autocatalytic cleavage and reassembly of pro-caspase-1 to form active caspase-1. Activated caspase-1 is responsible for cleavage of pro-IL-1 β , which once processed, will be in its biologically

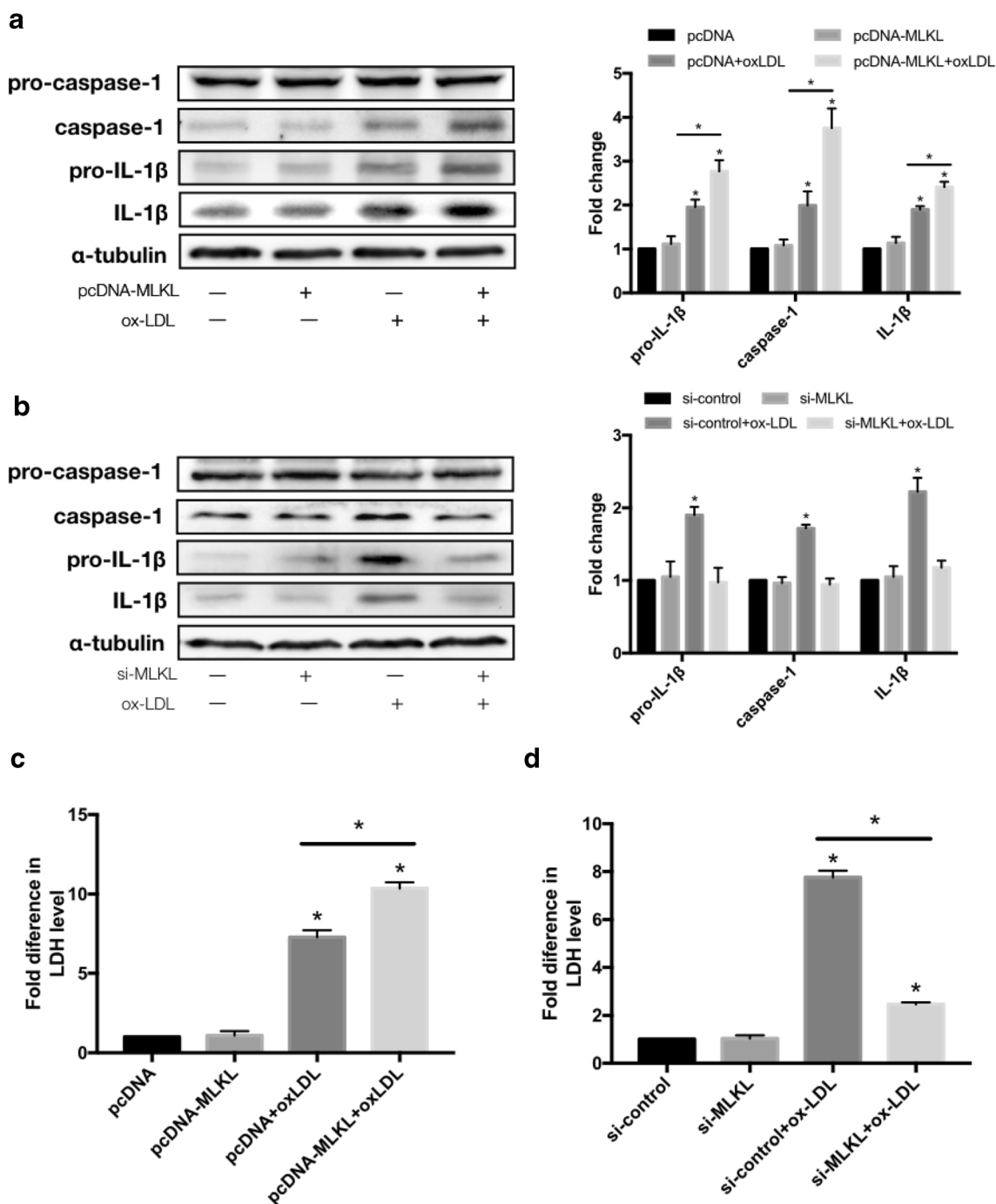


Fig. 2. MLKL triggered ox-LDL-induced pyroptosis in ECs. **a** ECs were transfected with pcDNA-MLKL (overexpressed MLKL) or pcDNA (pcDNA-mock) before ox-LDL treatment(50 mg/l, 24 h), and the protein levels of pro-IL-1 β , IL-1 β , and caspase-1 were measured by western blotting. **b** ECs were transfected with si-MLKL or si-control before ox-LDL treatment(50 mg/l, 24 h), and the protein levels of pro-IL-1 β , IL-1 β , and caspase-1 were measured by western blotting. **c** ECs were transfected with pcDNA-MLKL or pcDNA before ox-LDL treatment(50 mg/l, 24 h). Fold changes of LDH release compared with pcDNA treatment group. **d** ECs were transfected with si-MLKL or si-control before ox-LDL treatment(50 mg/l, 24 h). Fold changes of LDH release compared with si-control treatment group. * $P < 0.05$ versus the control group. Data are represented as mean \pm SD values from 3 independent experiments with each experiment done in triplicate.

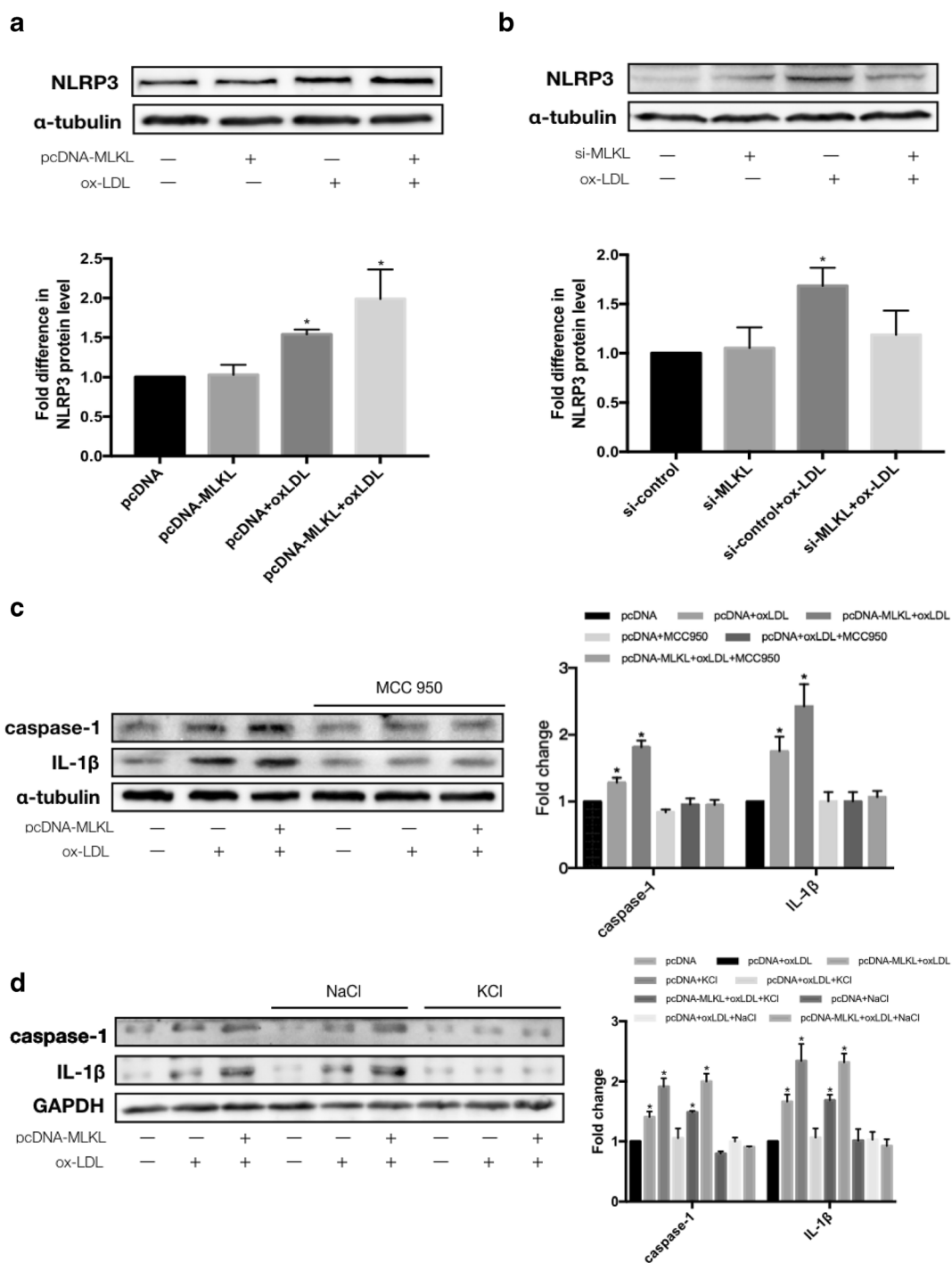


Fig. 3. MLKL triggered ox-LDL-induced pyroptosis *via* NLRP3 inflammasome activation. **a** ECs were transfected with pcDNA-MLKL (overexpressed MLKL) or pcDNA (pcDNA-mock) before ox-LDL treatment (50 mg/l, 24 h), and the protein levels of NLRP3 were measured by western blotting. **b** ECs were transfected with si-MLKL before ox-LDL treatment (50 mg/l, 24 h), and the protein levels of NLRP3 were measured by western blotting. **c** ECs were treated with NLRP3 specific inhibitor MCC950, and the protein levels of caspase-1 and IL-1 β were measured by western blotting. **d** Cell culture media were added with 50 mM potassium chloride and 50 mM sodium chloride respectively, and the protein levels of caspase-1 and IL-1 β were measured by western blotting. * $P < 0.05$ *versus* the control group. Data are represented as mean \pm SD values from 3 independent experiments with each experiment done in triplicate.

active form ready to be released from endothelial cells, and contributes to endothelial cell pyroptosis. Considering that endothelial cells are the barrier between blood and vessel walls, ox-LDL-induced EC pyroptosis participates in the starting point of atherosclerosis injury.

Pyroptosis is executed by inflammasomes, and the most well-known inflammasome is NLRP3 inflammasome, which is thought to act as a bridge between lipid metabolism and inflammation caused by cholesterol crystals [27]. Once activated, the inflammasome forms a plate-like structure that triggers caspase-1, cytokine release, and pyroptosis [34]. Caspase-1 is expressed abundantly in atherosclerosis plaques [35, 36]. What is more, caspase-1 contents in vulnerable plaques and ruptured lesions increased significantly in patients who died from acute coronary events [37]. We confirmed that ox-LDL-induced caspase-1 activation and IL-1 β maturation depend on NLRP3 in ECs, which offers novel insight into the mechanisms of arterial intima injury and inflammation caused by ox-LDL.

Necrosis is a newly discovered programmed cell death process associated with inflammatory disease [17]. MLKL is critical for necrosis, which releases molecules within the cell, leading to an inflammatory response [38]. Recent studies have shown that MLKL expression increases in people with unstable carotid atherosclerosis [39]. And phosphorylation of MLKL, which plays an important role in necrosis, can be detected in advanced atherosclerosis [40]. Our previous works show that MLKL aggravated inflammatory response caused by ox-LDL and confirm that MLKL-inhibited autophagy was mainly due to the activation of the mammalian/mechanistic target of rapamycin (mTOR)-dependent signaling pathway [22]. It has been reported that MLKL could induce activation of NLRP3 in a cell intrinsic way, which requires oligomerization and association of MLKL with cellular membranes, and subsequent reduction in intracellular potassium concentration [41, 42]. Our results also showed that MLKL induced an increase in NLRP3 inflammasome activation caused by ox-LDL. After block cell potassium efflux, the activation of caspase-1 and IL-1 β was significantly attenuated. Furthermore, we put forward that MLKL triggered ox-LDL-induced NLRP3 inflammasome activation and cell pyroptosis for the first time.

Collectively, the role of MLKL in ox-LDL-induced NLRP3 inflammasome activation was demonstrated. What is more, our results also indicate the potential importance of MLKL in atherosclerosis, which may be used as both a

diagnostic and therapeutic tool for the treatment of atherosclerosis.

AUTHORS' CONTRIBUTIONS

All authors contributed to the study conception and design. Qian Wu and Xin He designed the study, performed most part of the experiments, analyzed and interpreted the data, and wrote the manuscript. Li-Mei Wu, Ru-Yi Zhang, and Li-Min Li performed part of the experimental procedures. Chang-Meng Wu, Yuan-Bin Lu, Bing Hu, Chao Shi, Zhi-Feng Lu, and Biao Yang contributed to data acquisition. Lei Zheng, Yan-Wei Hu, and Qian Wang provided financial support and guided the completion of the project. All authors read and approved the final manuscript.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

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